

# Mutagenicity of the Halogenated Olefin, 2-Bromo-2-chloro-1,1-difluoroethylene, A Presumed Metabolite of the Inhalation Anesthetic Halothane

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The presumed halothane metabolite, 2-bromo-2-chloro-1,1-difluoroethylene, produces both base substitution and frameshift mutations in *Salmonella typhimurium*. Direct mutagenesis of isolated DNA also was observed by using a *Bacillus subtilis* transformation assay to score the production of mutagenic lesions in transforming DNA.

## Introduction

The discovery that the halogenated olefin, vinyl chloride, is carcinogenic (1-3) prompted investigations into the biological activities of this and other structurally related compounds. As a result of these studies two additional halogenated olefins, vinylidene chloride, which like vinyl chloride is used in large volumes in the synthesis of plastics, and trichloroethylene, used primarily as an industrial degreasing agent, have recently been identified as carcinogens and mutagens (4-7).

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a commonly used inhalation anesthetic. As much as 25% of the halothane absorbed during the course of anesthesia is metabolized (8) and the metabolic scheme shown in Fig. 1 was recently suggested by Cohen et al. (9) to explain the formation of the three major urinary metabolites identified in man. The proposed dehydrofluorination intermediate,

2-bromo-2-chloro-1,1-difluoroethylene (BCD), is of particular interest because of its structural similarity to the carcinogenic haloalkenes. In this regard there has been a growing concern, based on epidemiological studies (10, 11), that chronic, low-dose exposure, among anesthesia personnel, to inhalation anesthetics may be associated with an increased incidence of cancer, spontaneous miscarriages and births involving congenital anomalies.

In this communication we report the results of experiments in which BCD was tested for its capacity to induce mutations in two independent assays. The first of these, the Ames *Salmonella* auxotroph reversion test, utilizes a set of *S. typhimurium* strains which have been modified specifically for use in mutagen screening (12). Of the known carcinogens which have been tested in this system, 85% are detectable as mutagens while the incidence of false-positives is less than 10% (4). The second assay utilizes a transformable strain of *Bacillus subtilis* to score the formation of mutagenic lesions produced by interaction of test compounds with isolated transforming DNA. In this assay it is possible to determine whether the test compound is capable of direct reaction with DNA independent of any cellular metabolism.

## Materials and Methods

BCD was obtained from the Columbia Organic Chemicals Co., Columbia, S. C. It was estimated by the manufacturer, PCR Inc., Gainesville, Fla., to be 98.2% pure by gas chromatographic analysis.†

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†We recently were informed by Dr. V. Simmons of the Stanford Research Institute, that the preparation of BCD used in this study was only about 63% pure. Approximately 30% of the preparation consisted of  $\text{CF}_2\text{CH}_2\text{Cl}$ , one of the intermediates in the chemical synthesis of BCD, and approximately 5% consisted of a mixture of unidentified compounds. All of these compounds co-chromatographed on the column used by the manufacturer. This finding raises serious questions regarding which of the above compounds is responsible for the mutagenic activity we observed.

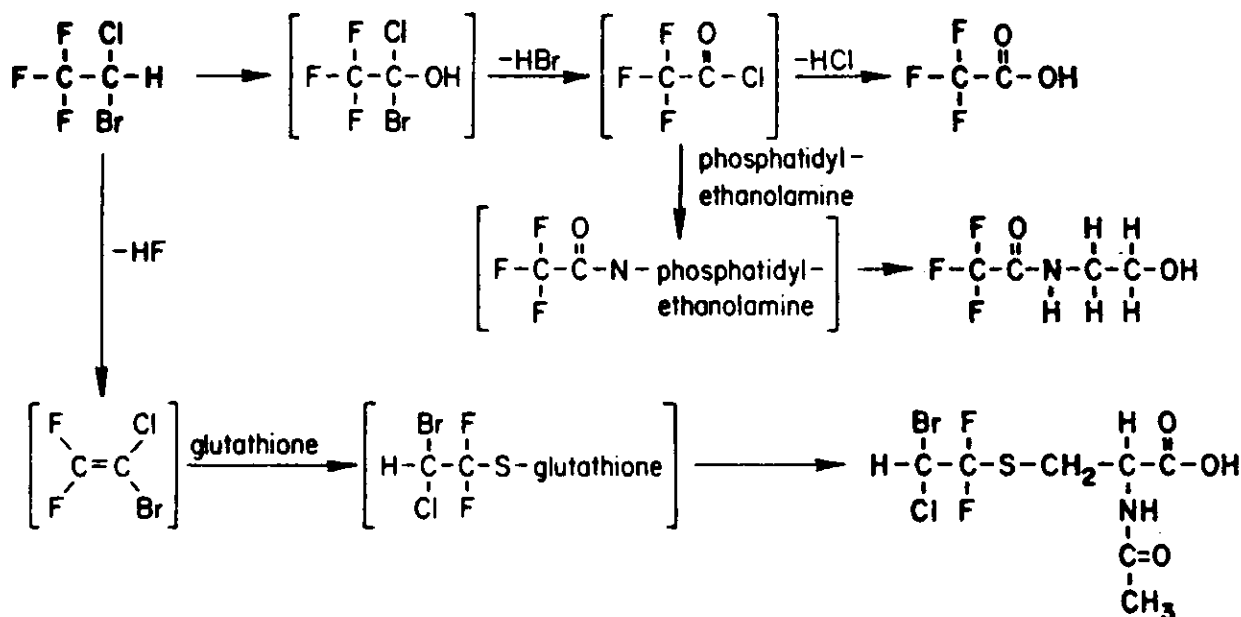


FIGURE 1. Proposed pathways of halothane metabolism according to Cohen et al. (9). The metabolic intermediates shown were suggested on the basis of the identified urinary metabolites: trifluoroacetic acid; *N*-trifluoroacetyl-2-aminoethanol, and *N*-acetyl-*S*-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine.

The principal contaminant is thought to be bromodifluoroacetylchloride which can form by spontaneous oxidation and rearrangement of BCD. The suggestion that bromodifluoroacetylchloride is the principal contaminant is supported by infrared spectral analysis which shows the presence of a trace amount of a carbonyl compound in the BCD preparation.

The *Salmonella* tester strains used in the mutagenesis assays require histidine for growth and are reverted to histidine-independence either by base-substitution mutagens (strains TA92, TA100 and TA1535) or by frameshift mutagens (strains TA98, TA1537 and TA1538). Strains TA100 and TA98 are derivatives of TA1535 and TA1538 which carry an R factor that interferes with accurate repair of damaged DNA and enhances the sensitivity of these strains to the mutagenic effects of some carcinogens (12). TA92 carries this same R factor but differs from TA100 and TA1535 in having a wild type cell envelope and is thus less permeable to some mutagens than TA100 and TA1535; it is also wild type for excision repair of DNA damage which is defective in TA100 and TA1535. BCD was tested for mutagenic activity on aliquots of the bacteria growing exponentially in tryptone broth, buffered to pH 7.4 with 0.05M sodium phosphate. The BCD was diluted in ethyl ether and the BCD-bacterial suspensions were incubated in screw-capped glass vials at 26°C for 30 min. The numbers of histidine-independent (*His*<sup>+</sup>) revertants in the reaction mix-

tures were determined by streaking samples on minimal plates lacking histidine. Total surviving cells were determined by plating on Difco tryptose blood agar base medium.

Direct mutagenic activity of BCD on isolated DNA was assayed by scoring for the induction of asporogenous (*Spo*<sup>-</sup>) mutants in cultures of *B. subtilis* which had been transformed with BCD-treated DNA. The DNA used was isolated by the Marmur method (13) from a *Spo*<sup>+</sup>, rifampin-resistant (*Rif*<sup>r</sup>) strain of *B. subtilis*. The genetic locus for *Rif*<sup>r</sup> is tightly linked to a cluster of genes, mutations in many of which lead to a *Spo*<sup>-</sup> phenotype (14). The isolated DNA was alkali denatured and treated at a final concentration of 74 µg/ml, with BCD in the presence of 7.2M dimethylformamide, 0.14M phosphate pH 7 at 53°C. The dimethylformamide and elevated temperature were used to minimize intrastrand base pairing thereby exposing the bases for reaction with the potential mutagen. The reaction mixture was incubated in a screw-capped glass tube and then dialyzed against 1000 volumes of 0.15M NaCl, 0.005M sodium phosphate, pH 7.2. The *Spo*<sup>+</sup> *Rif*<sup>r</sup> recipient cells were grown to competence (15) concentrated 10 fold and stored frozen in 10 per cent glycerol at -70°C until use. The cells were exposed to BCD-treated and control DNA under conditions optimized for transformation with single-stranded DNA (16) and then were plated on sporulation medium (17) containing 10 µg/ml rifampin which selects for *Rif*<sup>r</sup> transformants. Colonies

of Spo<sup>-</sup> mutants in the Rif<sup>r</sup> population were readily differentiated from those of Spo<sup>+</sup> cells since the former are colorless after 96 hr incubation on this medium, whereas the Spo<sup>+</sup> colonies accumulate a dark brown pigment. Since the loci specifying Rif<sup>r</sup> and Spo<sup>-</sup> are tightly linked and may be carried on a single piece of transforming DNA, selection for Rif<sup>r</sup> enhances the possibility of detecting cells which have incorporated a DNA fragment that received a mutagenic hit in a sporulation-specific gene.

This assay is analogous to another described by Freese and Strack (18) which utilized markers in the tryptophan (Trp) biosynthetic pathway for detecting DNA-reactive mutagens. The advantage of scoring for mutants in the sporulation rather than the tryptophan pathway is the relative sensitivity with which the Spo<sup>-</sup> mutants can be detected. A single Spo<sup>-</sup> clone is observable against a background of up to 1000 Spo<sup>+</sup> colonies whereas it is difficult to detect the Trp<sup>-</sup> mutants, which excrete a metabolite that fluoresces under ultraviolet light, if the background of Trp<sup>+</sup> colonies exceeds 100 to 200 per plate.

## Results and Discussion

BCD induced both base substitution and frameshift mutations reverting *Salmonella* strains TA92, TA98, and TA100 (Fig. 2). Since all of these are R factor carrying strains the induction of both types of mutations may be a consequence of error prone repair of BCD-damaged DNA. The presence of the wild type cell envelope decreased sensitivity to BCD mediated mutagenesis. BCD at the concentrations used in the above experiments did not kill the cells. Toxicity as well as an increased mutagenic effect was observed at higher concentrations (Fig. 3). From its structure BCD is expected to be highly lipophilic. The concomitant increase in both toxicity and mutagenesis may reflect BCD mediated damage to cellular membranes resulting in an increase in permeability to the mutagen. The increased mutation frequency, i.e., the number of revertants per viable cell observed at the higher BCD concentrations is not due simply to plating lower numbers of viable cells on the selective plates. While it is known that the numbers of revertants which arise spontaneously during colony formation are approximately constant over a plating range of 10<sup>4</sup> to 10<sup>8</sup> cells, it is possible to correct for these spontaneous "plate" mutants when calculating the induced reversion frequency by subtracting the numbers of His<sup>+</sup> revertants observed in the untreated samples (19).

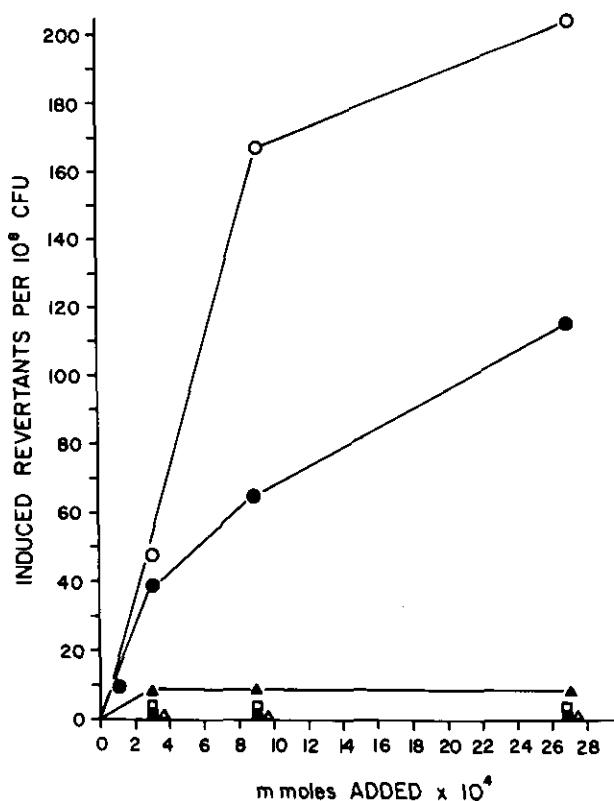


FIGURE 2. BCD induced reversion of *S. typhimurium* strains: (Δ) TA92; (○) TA98; (●) TA100; (■) TA1535; (◻) TA1537; (◊) TA1538. The numbers of induced revertants (19) were calculated as [(His<sup>+</sup> revertants/BCD treated sample)-(His<sup>+</sup> revertants/untreated sample)]/(survivors/treated sample).

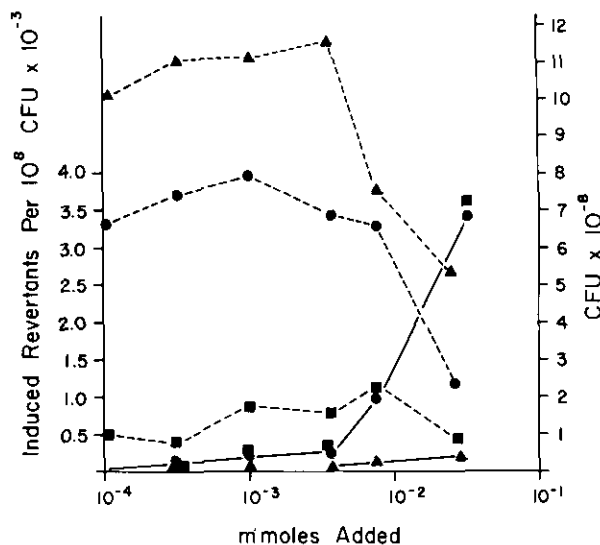


FIGURE 3. BCD mediated mutagenicity and toxicity on *S. typhimurium* strains: (Δ) TA92; (■) TA98; (●) TA100; (—) Revertant colony forming units (CFU), (---) Viable cells.

**Table 1. BCD-mediated mutagenesis and inactivation of transforming activity of *B. subtilis* DNA.<sup>a</sup>**

Incubation time, min	BCD added, mmoles × 10 <sup>2</sup>	Rif <sup>r</sup> transformants/ml × 10 <sup>-3</sup>	Spo <sup>-</sup> /Rif <sup>r</sup> colonies × 10 <sup>4</sup>
0	0	104	5
15	4.8	26	33
30	9.6	8	112
45	14.4	1	344
60	19.2	1	1,005

<sup>a</sup>The DNA was reacted with BCD as described under Methods. The initial reaction volume was 3.6 ml. At each time point a 0.5 ml sample was removed and 0.05 ml of a 0.96M solution of BCD in ethyl ether was added. The DNA was dialyzed and added at a final concentration of 5.5 µg/ml to competent cultures of *B. subtilis* Rif<sup>r</sup> Spo<sup>+</sup>. The transformation mixture was incubated at 37°C for 1 hr then diluted 5-fold into nutrient broth and incubation continued for 3 hr prior to plating to allow expression of the Rif<sup>r</sup> phenotype.

Increased numbers of Spo<sup>-</sup> mutants were observed among the cells transformed to Rif<sup>r</sup> with BCD-treated DNA (Table 1). Inactivation of transforming activity also was observed and further studies will be required to determine if mutagenesis and inactivation are due to the same type of lesion in the DNA. Distinct mutagenic and inactivating effects have been observed in a similar assay with the mutagenic hydroxylamines (20).

Microsomal activation was not required for mutagenesis either with *Salmonella* or isolated DNA. Thus BCD differs from most of the other mutagenic olefins studied which require metabolism, presumably to their respective oxiranes for activity (6, 21). Vinyl chloride also has been shown to be directly mutagenic for *Salmonella* (22-24), but this compound has not been tested for direct mutagenesis on isolated DNA. The direct mutagenic effect of vinyl chloride on *Salmonella* may be due to free radical formation (24). The mutagenic effect of BCD may result from direct reaction with nucleophilic sites on DNA bases. From the urinary metabolites of halothane which have been identified it appears that BCD can react directly with nucleophiles such as glutathione (9). As noted under Methods, we have been informed that BCD may spontaneously oxidize to form an acylhalide. This oxidation product may contribute to the observed mutagenesis if present or formed in high enough concentrations during the mutagenesis assays. It is not known if this oxidation product is formed in significant amounts *in vivo* but there are several minor urinary metabolites of halothane which have not been identified (9). There are also indications from *in vitro* studies that there is more than one halothane metabolite which is capable of binding to macromolecules (25).

The biological significance of BCD mutagenicity with respect to the specific morbidities noted in the epidemiological surveys of anesthesia personnel is not known. As yet neither ourselves nor others have been able to observe halothane mediated

mutagenicity even in assays which included an *in vitro* metabolic activation system (26). It has recently been reported that the dehydrofluorination of halothane which would give rise to BCD occurs only under low O<sub>2</sub> tension (25). Thus prior failures to observe halothane mediated mutagenesis may have been related to inappropriate *in vitro* metabolic activation conditions. In this regard the present study should be of use in determining the minimal levels of halothane-derived BCD which can be detected in standard mutagenesis assays.

This investigation was supported by research grant No. BC-200 from the American Cancer Society. The authors thank Ms. V. Lima for her technical assistance and the chemists at Columbia Organic Chemicals and PCR for their helpful discussions.

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